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Immune response and survival of a leafcutter ant with a compromised immune system during an entomopathogenic challenge

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Abstract

Social insects employ diverse strategies, including individual and collective sanitary care as well as colony-level hygiene, to prevent, manage and control pathogen transmission. The immune system constitutes a key component of the individual defensive repertoire, but its activation entails trade-offs with other life history traits due to its high metabolic cost. In this study, we investigated how different immune strategies interact in social insects. We challenged workers of the leaf-cutting ant *Atta cephalotes* with an entomopathogen to compare hygienic behavior and survival rates in the presence and absence of a previously activated immune encapsulation response. Entomopathogenic-challenged ants: (a) increased the production of infrabuccal pellets, formed by collecting detritus and fungal conidia and mixing them with antimicrobial compounds as a prophylactic behavior to avoid conidia germination; and (b) exhibited a reduced encapsulation response. Although the encapsulation response of insects is typically initiated within a few hours after infection, in our experiment neither pellet production nor encapsulation response depended on the timing of the prior immune challenge. Moreover, simultaneous activation of the immune system with both a foreign body (nylon implant) and entomopathogenic fungal conidia significantly reduced survival rates. Our findings suggest that when the ant immune system is chronically challenged to encapsulate a foreign body, its defensive capacity against a fungal pathogen is weakened.

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Keywords
Atta cephalotes, Attini, encapsulation, fungal infection, hygiene behavior, *Metarhizium*

Introduction

Many social insects exhibit broad geographic distributions and frequently occur in colonies with high population densities (Wilson 1990; Hölldobler and Wilson 2009). Colonies of social insects may contain large numbers of individuals with high rates of physical contact, creating favorable conditions for pathogen transmission (Schmid-Hempel 1998). Moreover, genetic homogeneity within colonies facilitates the spread of disease (Hamilton 1987; Seeley and Tarpy 2007), while high colony density may also promote the evolution of resistance to host disease management strategies (Schmid-Hempel 2021). Preventing disease outbreaks in social insects relies on a combination of behavioral and physiological defenses, collectively termed social immunity (Cremer et al. 2007; Casillas-Peréz et al. 2023). These defenses include behaviors such as mutual grooming to remove pathogens, secretion of antimicrobials from specialized glands, disposal of waste and infected brood, and collective actions such as social fever and nest cleaning, all of which work together to limit disease transmission within a colony (Hughes et al. 2002; Farji-Brener et al. 2016). In addition to these social strategies, the individual immune system provides defense against pathogens through both cellular and humoral responses (Cotter and Kilner 2010). The humoral immune response in insects involves the production of antimicrobial peptides and enzymes, such as lysozymes and phenoloxidase, which circulate in the hemolymph to neutralize or destroy pathogens. The cellular response, by contrast, is mediated by hemocytes, which recognize invaders and either engulf them via phagocytosis or encapsulate larger threats. Encapsulation is a key defense against pathogens too large to be phagocytosed, like parasitoid eggs or implanted materials. In this process, multiple hemocytes surround the foreign body, forming a melanized capsule that effectively isolates and kills it (Hilyer 2016; Ali et al. 2020).

The functional costs and interactions between social and individual immunity are not yet well characterized. The individual energetic budget of a social insect is finite (Moret and Schmid-Hempel 2000), and activating the immune response incurs a metabolic cost that may have physiological consequences if the defensive system becomes compromised (Freitak et al. 2003). In this study, we investigated how the functional costs associated with individual immunity interacts with those of collective behavioral immunity in social insects. Our first hypothesis is that there is a trade-off between individual immune responses and collective hygienic behaviors. We predict that increased investment in collective behaviors, such as allogrooming, will be associated with reduced individual immune responses, including mechanisms such as encapsulation and melanization. To test this hypothesis, we challenged the individual immune system of workers of the leaf-cutter ant

species *Atta cephalotes* (Formicidae, Attini) by implanting a small nylon filament into the body cavity, which provokes an encapsulation and melanization response (Vilmos and Kurucz 1998; Ardia et al. 2012). Subsequently, the ants were exposed to conidia of an entomopathogenic fungus at different time points post-implantation. After 24 hours, we measured the strength of the encapsulation response around the implant and quantified the number of infrabuccal pellets produced, using this as an indicator of collective grooming behavior. Infrabuccal pellets are compacted masses of detritus and pathogenic conidia collected during grooming and formed within the infrabuccal cavity of ants. When *Atta* workers are exposed to pathogenic fungi, these pellets are treated with antimicrobial secretions from the metapleural glands prior to being discarded in waste piles, thereby reinforcing their collective hygienic response (Little et al. 2003; Fernández-Marín et al. 2006; Fernández-Marín et al. 2015).

Our second hypothesis is that compromising the individual immune system increases susceptibility to pathogenic infection. We predict that challenging the immune system with a foreign object (e.g., a nylon implant) will reduce the ants' ability to mount an effective immune response against entomopathogenic fungal infection. In a second experiment, we challenged the immune system of *A. cephalotes* workers with a nylon implant while simultaneously exposing them to an entomopathogenic fungus. We then compared the survival rates among three groups: i) ants with both the implant and fungal exposure; ii) ants exposed only to the fungus; and iii) ants with only the nylon implant. Testing both hypotheses elucidates the trade-offs between individual and collective immune responses in ants, highlighting the energetic and physiological costs associated with activating multiple defenses simultaneously. Understanding these interactions may provide insights into how energy constraints and pathogen pressure have shaped the evolution of cooperative disease management in social insect societies.

Materials and methods

Colony collection and maintenance

We collected 3–6 chambers of ten mature colonies of the leaf-cutter ant species *A. cephalotes* in secondary forest located in Soberania National Park, near Gamboa, Colón Province, in the Republic of Panama. The colonies were kept under laboratory conditions using standard methods (Weber 1972) in the facilities of Smithsonian Tropical Research Institute in Gamboa. All colonies were maintained in the laboratory conditions for about a week before being used for experiments.

Entomopathogenic Fungi: We used dry conidia from the generalist entomopathogenic fungi species *Metarhizium brunneum* and measured survivorship rates and hygienic behavior (following Fernández-Marín et al. 2015; Bonadies et al. 2019).

Experiment 1: Fungal grooming behavior and immune system responses to a fungal pathogen by ants with a previously challenged immune system

For this experiment, we used seven *A. cephalotes* colonies collected between January and April 2017. From each colony, we took 60 medium-size workers (head width mean = 2.04 mm, SD = 0.18). Fungal grooming behavior is defined as the removal of fungal conidia from infected areas by worker ants. The conidia are gathered in the infrabuccal pocket, where they accumulate and are formed into pellets before being discarded. These infrabuccal pellets produced were counted following Little et al. (2003) and Fernández-Marín et al. (2006). To elicit an immune response, we inserted a transparent nylon filament into the body of the ants. The filament becomes coated by immune cells, and its subsequent darkening through melanization was used as a measure of the immune reaction. The implantation procedure followed Baer et al. (2006), Cotter et al. (2004), and Couceiro et al. (2016) with slight modifications. Using a stereomicroscope and a sterile hypodermic needle, we punctured the intersegmental membrane between abdominal tergite IV and V of an immobilized worker. A sterile piece of transparent nylon (diameter of 0.13 mm and a length of ~0.4 mm; mean = 0.44, SD = 0.05) was then inserted into the gaster using sterile fine forceps. After implantation, we established sub-colonies composed of ants with nylon implants from the same original colony. Each sub-colony consisted of 20 workers, 1.5 g of the symbiotic fungus garden placed in the center, and pieces of moistened paper towel placed on the edge of the Petri dish. For the experimental treatments, each sub-colony was inoculated with approximately 4.6×10^6 *Metarhizium brunneum* conidia, which were collected with sterile tweezers on a piece of Parafilm and subsequently transferred to the sub-colonies. Inoculations were carried out at two different time points—one for each treatment—within a 24-hour period.

i. "Treatment 0 hour" – Sub-colonies were inoculated with *M. brunneum* immediately after they were established. Three hours later, we collected the infrabuccal pellets and plated them on Petri dishes with potato dextrose agar (PDA) to assess germination rates. After sampling, ants were transferred to a new petri dish free of *M. brunneum* with fresh fungal garden, and moistened paper towel, where they remained for the next 21 hours to complete the 24-hour period. At 24 hours, the ants were sacrificed.

ii. "Treatment 21 hours" – Sub-colonies were left undisturbed for 21 hours after establishment, and then were infected with *M. brunneum* conidia. At 24 hours, we collected infrabuccal pellets and sacrificed the ants. Pellets were placed on PDA as described above.

iii. "control" – Sub-colonies received no *M. brunneum* conidia inoculation and were left undisturbed for 24 hours, after which the ants were sacrificed.

We removed nylon implants from all the sacrificed ants using sterile fine forceps. Each implant was photographed under a stereomicroscope (Nikon SMZ800) equipped with a camera (Nikon DS-Fi1). Using Image J version 1.50b (Schneider et al. 2012) we measured implant size and mean grayscale values. Grayscale represents light intensity im-

ages, ranging from 0 (darkest) to 255 (white). All statistical analyses were performed in R software (R Development Core Team 2018). We used a Wilcoxon rank test with a (95% confidence level) to compare pellet production and germination rates among treatments. To evaluate treatment effects on the encapsulation response, we applied generalized linear mixed models (GLMMs) using the function lmer in the lm4 package (Bates et al. 2015), with mean grayscale value as the response variable, "treatments" with the levels 0 hour, 21 hours and control as fixed effect and "colony of origin" as a random effect. To compare between the means from different treatments, we used a Least Significant Difference (LSD) *post-hoc* pair-wise comparison in the multcomp package (Hothorn et al. 2008).

Experiment 2: Survival with a compromised immune system

For this experiment, we used three *A. cephalotes* colonies. From each colony, we established six groups of 10 ants, each provided with 1.5 g of fungus garden in Petri dishes. Within each colony, we tested three treatments: (1) ants with a nylon implant inserted in the gaster (as described in Experiment 1); (2) wounded ants that received only a membrane puncture without implant; and (3) intact ants with neither a puncture nor an implant. Each treatment was further divided into two groups: one exposed to *M. brunneum* conidia, and one unexposed control.

To infect ants, we used a 0.05% Tween 20 solution containing 1.3×10^4 conidia mL^{-1} of *M. brunneum.* Each ant was dipped individually into the solution using sterile forceps and then placed on filter paper to remove excess liquid. Control groups were treated identically but dipped into Tween 20 solution (0.05%) without conidia. Ants were kept in petri dishes with ~0.4 grams of symbiotic fungi, and moistened paper. The ants were monitored daily for 10 days, and mortality was recorded. Dead ants were surface-sterilized and placed in humid chambers to confirm *M. brunneum* infection. Individuals that did not develop *M. brunneum* growth were excluded from analyses. A pairwise Log rank test comparison was used to detect differences between survival curves from the survival R package (Therneau 2021). Mean values are reported with standard deviations.

Data sets used in all statistical analyses are available at https://smithsonian.figshare.com/articles/dataset/Immune_response_and_survival_of_a_leafcutter_ant_with_a_compromised_immune_system_during_an_entomopathogenic_challenge/30307537?file=58573795.

Results

There were no differences in pellet production comparing sub-colonies inoculated at 0 and 21 hours post-establishment (mean pellet production = 11.14 ± 3.8, and 11.42 ± 6.16, respectively; median pellet production = 11 and 9, respetively; Wilcoxon, $W = 26.5$, $p = 0.8$). Similarly, the number of germinated pellets did not differ between the two groups (mean pellet germination = 4.28 ± 4.78 and 3.28 ± 4.53, respectively; median pellet germination = 11 and 9; Wilcoxon, $W = 25.5$, $p = 0.9$; Fig. 1). Control sub-colonies did not produce any visible infrabuccal pellets during the experiment.

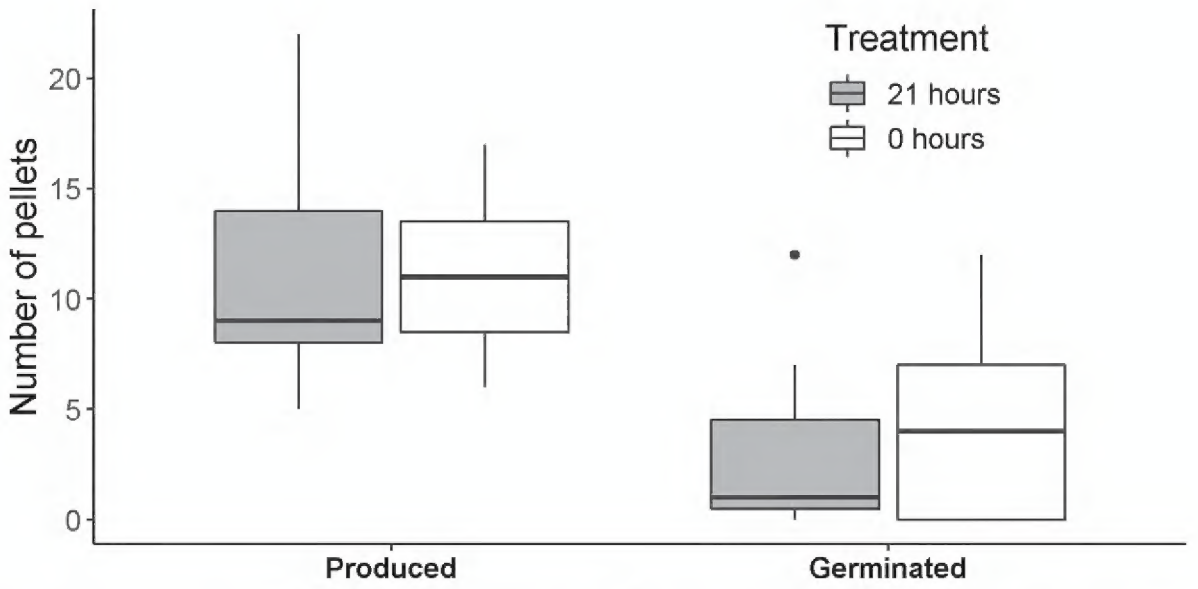

Figure 1. Pellets produced (left) and germinated (right) in subcolonies infected with *M. brunneum* at 0 and 21 hours. No significant differences were detected in pellet production or germination between ants inoculated at 0 and 21 hours. Control subcolonies did not produce any visible infrabuccal pellets.

Nylon implants from ants inoculated with conidia at 0 hours were the lightest (mean gray scale = 140.4 ± 28.66), while those from control ants were the darkest (mean gray scale = 131.06 ± 29.48). Exposure to *M. brunneum* conidia (0 hour, 21 hours and control) significantly affected mean gray scale ($x^2 = 6.2$, $p = 0.0467$). Implants from ants inoculated at 0 hours and 21 hours differed from the control (Fisher's LSD: 0 hour-control, $p = 0.028$; 21 hours-control, $p = 0.044$) but not from each other (Fisher's LSD: 0 hour-21 hours, $p = 0.84$) (Fig. 2).

In experiment 2, nylon-implanted, wounded and unwounded ants from control groups had higher survival over 10 days than any group exposed to *M. brunneum* (Fig. 3). Survival curves of control ants differed significantly from all conidia-exposed groups to *M. brunneum* in a pairwise comparison (log-rank, $p < 0.05$). Within the control group, survival did not differ among the curves of the three treatments (log-rank, $p > 0.05$). In contrast, within the group exposed to *M. brunneum* conidia, survival curves differed between nylon-implanted and unwounded ants (log-rank, $p = 0.023$) but not between nylon-implanted and wounded (log-rank, $p = 0.43$) nor between wounded and unwounded ants (log-rank, $p = 0.17$) (Fig. 3).

Discussion

Ants rely on both individual and social defenses against pathogens, employing hygienic prophylactic behaviors and immune defense to prevent or resist infections. Our results show that pellet production is independent of the timing of the initial immune challenge with a nylon implant during the first 24 hours. Grooming begins within minutes after conidia exposure, as previously reported (Fernández-Marín et al. 2006; Fernández-Marín et al. 2013). Preventing disease is less costly than treating it, which likely explains why grooming frequency does not decrease even when other defensive mechanisms are activated.

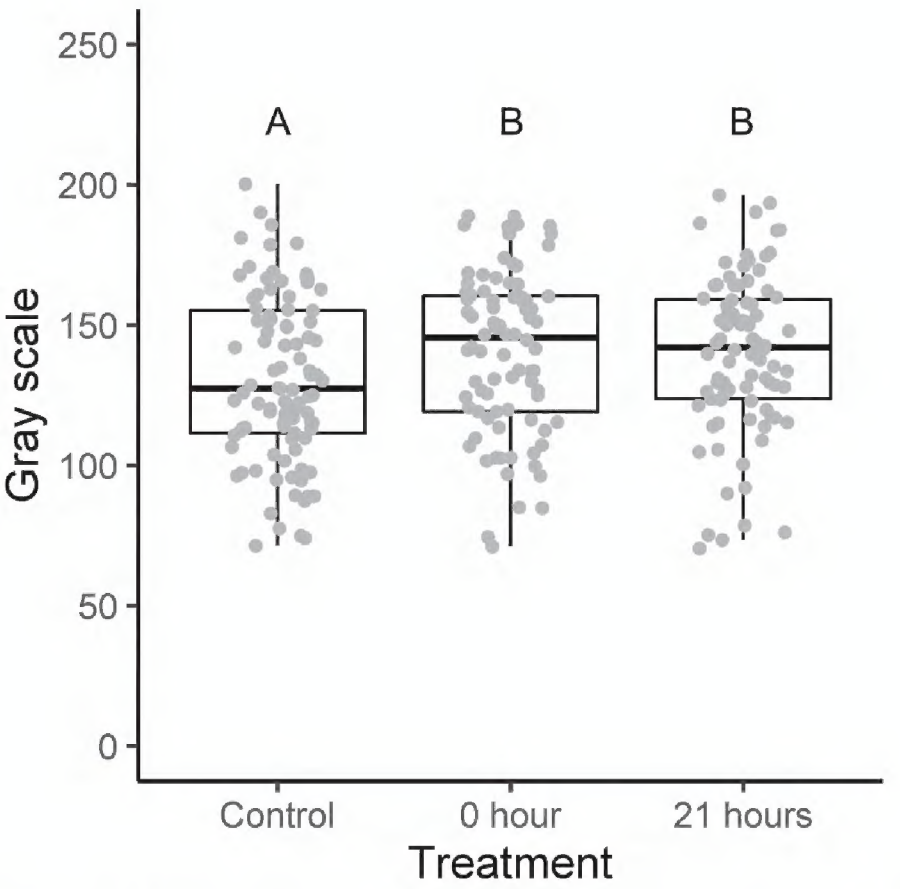

Figure 2. Boxplot of grayscale values measured from nylon implants across control and treatment groups inoculated with conidia at 0 and 21 hours. Different letters above the boxes indicate statistically significant differences ($p < 0.05$); groups sharing the same letter are not significantly different ($p > 0.05$). Lower grayscale values represent darker nylon, indicating greater encapsulation and melanization, and thus a stronger immune response. Conversely, higher grayscale values correspond to lighter nylon, reflecting reduced encapsulation and melanization, and therefore a weaker immune response.

Nylon implants and exposure to the entomopathogen *Metarhizium anisopliae* in *Atta cephalotes* have previously been shown to induce a stronger encapsulation response compared to a nylon-only control (Valencia-Giraldo et al. 2021). In our study, using *M. brunneum*, we found that *A. cephalotes* workers exhibited a weaker encapsulation response when exposed to conidia either 0 or 21 hours after implantation, relative to the nylon-only control. This difference in encapsulation may be related to the pathogen species used or to differences concentrations between studies. In either case, since we observed differences between the nylon-only control and conidia-exposed ants within a 24 hours period, immune mechanisms against conidia must act rapidly. Notably, ants exposed to conidia at 21 hours spent most of the 24-hour period without pathogen exposure, yet the control group displayed a stronger encapsulation response. This suggests that the observed difference likely arose from immune processes triggered during the final 3 hours, indicating that this short window was influential in shaping the overall immune outcome. Initiating social or innate immune responses to pathogens carries a physiological cost. In *Acromyrmex* ants, pathogen-fighting secretions from the metapleural glands account for approximately 13–20% of basal metabolism (Poulsen et al. 2002), and maintaining antibiotic-producing symbiotic bacteria on the cuticle can increase respiration rates by 10–20% (Poulsen et al. 2003). In other insects, compromising the immune system with a nylon implant has been shown to increase metabolic rate by about 8% (Freitak et al. 2003). In our study, ants with nylon implants

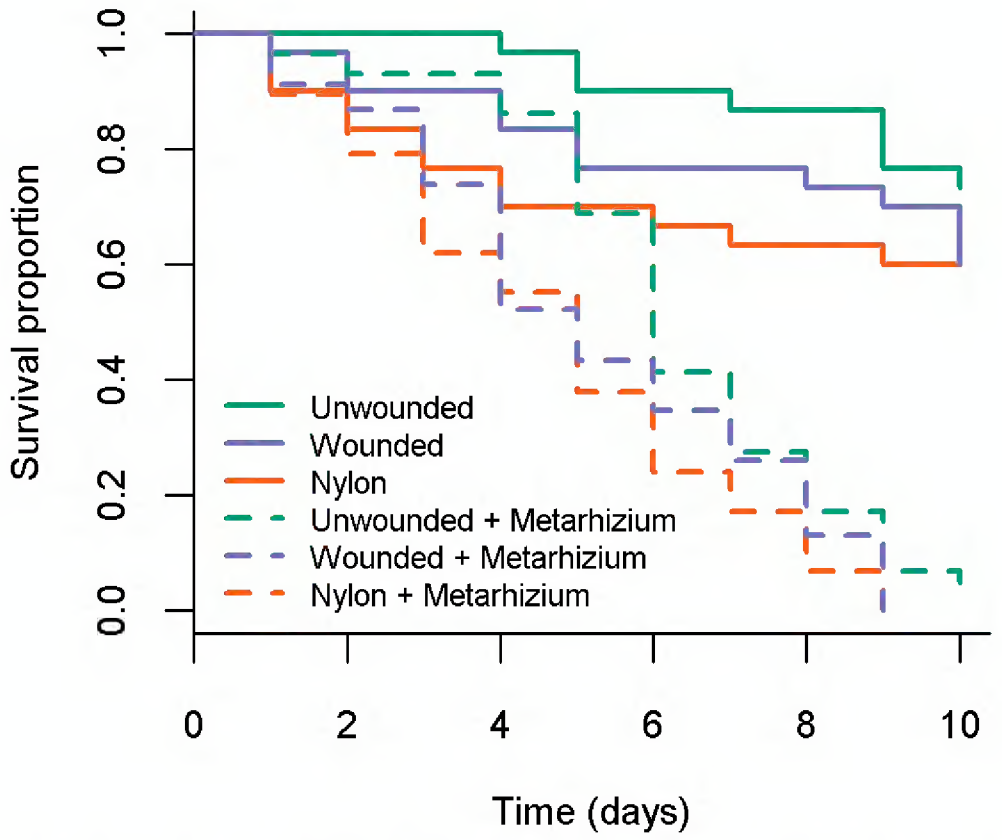

Figure 3. Cumulative survival proportion (Kaplan-Meier curves) from *A. cephalotes* workers.

that were also exposed to pathogenic conidia exhibited a weaker immune response than control ants, suggesting a possible trade-off between individual immunity and collective hygienic behaviors. Such trade-offs in resource allocation between internal immune responses and external antimicrobial defenses have also been observed in subsocial *Nicrophorus* beetles (Cotter et al. 2013). Simultaneous reliance on both innate and social immune strategies may reduce host performance due to high metabolic costs, with potential fitness consequences (Moret and Schmid-Hempel 2000). Previous studies with *Atta sexdens* demonstrated that ants with suppressed innate humoral immune systems exhibited reduced survival rates (Dornelas et al. 2017).

Our second experiment also showed reduced survival in ants with compromised immune systems when inoculated with *Metarhizium* conidia. Individuals with challenged immune systems are less likely to resist subsequent infections, leading to lower survival rates and associated social costs (see Bashir-Tanoli and Tinsley 2014). Such trade-offs between individual and social costs also have evolutionary consequences, ranging from use of antimicrobial compounds (Fernández-Marín et al. 2015) to behavioral adaptations (Starks et al. 2000; Sun and Zhou 2013; Stroeymeyt et al. 2014; Bos et al. 2015). Moreover, cellular immune responses have shown to decrease in groups with larger number of individuals compared to related groups with fewer individuals (López-Uribe et al. 2016), presumably due to the greater efficacy of social immunity. Additional variation may be linked to differences in number of immune-related genes across some social insect lineages (Evans et al. 2006; Suen et al. 2011). In addition to the complementarity observed between individual and social immunity (Masson et al. 2024), it is important to consider how the metabolic costs of one or several defense strategies may constrain overall immune capacity, thereby compromising defensive effectiveness.

In this study, we identified trade-offs between innate and social immune defenses in a social insect. Clarifying these trade-offs is essential for understanding how evolutionary processes allocate resources for pathogen defense during the emergence of complex societies. Looking ahead, the diversity of attine social organization offers a valuable framework to explore how such trade-offs influence both individual and collective immunity in relation to colony size and social complexity.

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